Repair of spinal cord transection and its effects on muscle mass and myosin heavy chain isoform phenotype

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ALTHOUGH AN EFFECTIVE STRATEGY for repairing injured spinal cords still remains elusive, significant advances have evolved during the past two decades in at least three critical areas: 1) identifying and neutralizing molecules that inhibit axonal regeneration (13, 26), 2) mitigating scar formation (1, 2), and 3) developing scaffolds that bridge the lesion site and promote axonal regeneration (15, 21). With respect to this latter approach, Cheng et al. (7) developed a peripheral nerve graft approach, which use to evaluate the myosin heavy chain (MHC) isoform expression in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Animal model and experimental manipulation. Twenty-four adult female Sprague-Dawley rats (225–250 g; Harlan, San Diego, CA) were randomly divided into three groups: 1) sham control group (n = 8), 2) spinal cord transection (Tx; n = 10), and 3) spinal cord transection plus peripheral nerve graft repair (Tx+PNG; n = 9). Two animals in the Tx group and one animal in the Tx+PNG group died prematurely. At the end of the protocol, there were eight animals in each group involved in all experimental procedures. All procedures involving animals followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of University of California, Irvine. Animals were housed in ventilated humidity- and temperature-controlled (23–25°C) rooms with a 12:12-h light-dark cycle.

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Before surgery, all animals were anesthetized using ketamine (70 mg/kg) and xylazine (8 mg/kg). Animals were maintained on a heating pad, and the rectal temperature was monitored and maintained within 3°C of normal temperature during surgery. Bipolar electrocoagulation was used to minimize bleeding in some animals. Animals in the sham control group underwent a laminectomy only (T8 level). In the Tx group, a laminectomy was also performed, followed by two complete transverse cuts of the spinal cord (at the T8 level), creating a gap of ~5 mm. A surgical microscope was used to ensure the complete removal of neural tissue, including fiber bundles, from the 5-mm gap. The muscle and skin layers were closed with 2-0 sutures. The Tx+PNG animals also underwent spinal cord transaction as described above, except the 5-mm gap was repaired using peripheral nerve autotransplantation and aFGF treatment. The peripheral nerve auto transplantation and aFGF procedures are the same as those described in previous work by our group (16). Briefly, this involved harvesting 18 intercostal nerve segments and transplanting them to bridge the 5-mm gap (white matter (proximal site) to gray matter (distal site)). A mixture of aFGF (1 μg; R&D Systems, Minneapolis, MN) in fibrin glue was applied on the top of grafts. The vertebral column was fixed in the dorsiflexion position by wiring with a compressive S-shaped monofilament surgical steel (B&K gauge 20, DS-20; Ethicon) loop fastened to the vertebral column with nonabsorbable threads. Following surgery, each animal in the Tx and Tx+PNG groups had their bladders expressed manually twice a day. Heating pads were applied beneath the cages during the first 3 days postsurgery. Animals were killed 6 mo following surgery.

**BBB open-field locomotion test.** Evaluation of gross motor behavior was conducted by BBB open-field locomotion test at 6 mo after surgery. The rats were placed in the middle of a circular enclosure made of molded plastic with a smooth, non-slip floor (90-cm diameter, 7-cm wall height). Each session lasted 4 min. The open-field locomotor activity score was assigned by observation and scoring behaviors involving the trunk, tail, and hindlimb. Scores ranging from 0 to 21 (0 = no movement, 21 = normal movement) were used. All examiners were blinded regarding animal groups. After the behavioral test, each animal was anesthetized and both soleus muscles were harvested for the studies described below. The animal was then euthanized and perfused with 4% paraformaldehyde (PFA) and incubated overnight at 4°C. After three rinses in PBS, sections were exposed to a biotinylated secondary antibody (1:200; Vector, Burlingame, CA) followed by the ABC Elite kit (Vector) for 1 h each, and then the reaction was visualized by treatment with 0.02% 3,3’-diaminobenzidine with 0.001% H2O2 in Tris-saline for 2–6 min.

**Quantitative assessment of 5-HT fibers below the lesion site.** The number of 5-HT-positive fibers (>150 μm in length) in the graft and caudal to the graft site were counted by two blinded observers under bright-field conditions. The chosen sections were separated by 60 μm to avoid counting the same fiber twice. Approximately 10 sections from each animal were analyzed.

**Evaluation of gross motor behavior.** The initial body weights of the animals in the sham control, Tx, and Tx+PNG groups were 238 ± 3, 235 ± 1, and 241 ± 4 g, respectively. Six months following spinal cord surgery, each group had gained body weight (see Table 1); however, the mean weights of the Tx and

**RESULTS**

**Body and muscle weights.** The initial body weights of the animals in the sham control, Tx, and Tx+PNG groups were 238 ± 3, 235 ± 1, and 241 ± 4 g, respectively. Six months following spinal cord surgery, each group had gained body weight (see Table 1); however, the mean weights of the Tx and

**Table 1. Body weight and soleus muscle weight 6 mo after spinal cord transection**

<table>
<thead>
<tr>
<th></th>
<th>Sham Control</th>
<th>Tx</th>
<th>Tx+PNG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>291±5</td>
<td>263±5*</td>
<td>259±5*</td>
</tr>
<tr>
<td>Soleus weight, mg</td>
<td>125±3</td>
<td>81±3*</td>
<td>96±4†</td>
</tr>
<tr>
<td>Soleus/body weight ratio, mg/g</td>
<td>0.44±0.01</td>
<td>0.31±0.02*</td>
<td>0.37±0.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Tx, spinal cord transection; Tx + PNG, spinal cord transection and repair (peripheral nerve graft). *Significantly different compared with sham control. †Significantly different compared with Tx.
Tx+PNG animals were ~3–4% less than that of the sham control group. As expected, Tx produced a large loss in soleus muscle weight (~40% compared with the sham control group; \( P \leq 0.05 \); see Table 1). Although the soleus muscle weight in the Tx+PNG group was also significantly less than that of the sham control group, it should be noted that it was significantly larger (19%; \( P \leq 0.05 \) ) than that of the Tx group.

Muscle fiber cross-sectional area. As shown in Fig. 1, spinal cord transection produced a large loss in muscle fiber cross-sectional area (B and D). The mean cross-sectional area of the Tx soleus muscle fibers was \( \sim 2,800 \mu m^2 \) less than that of the sham control fibers, representing a loss of ~60% in muscle fiber cross-sectional area. Repair of the spinal cord injury was effective in partially restoring muscle fiber cross-sectional area, given that the mean cross-sectional area of muscle fibers in the Tx+PNG group was ~55% greater than that in the Tx group (\( P < 0.01 \)).

Single muscle fiber MHC isoform composition. The majority (>90%) of single fibers in the sham control soleus muscles expressed only the slow type I MHC isoform (Figs. 2A and 3A). Spinal cord transection markedly altered this pattern of MHC expression such that there were very few slow type I fibers (\( \approx 1\% \); Figs. 2B and 3B). This loss in slow type I fibers was accompanied by the appearance of large pools of fast type IIA fibers, fast type IIIX fibers, and hybrid fibers (i.e., I/IIX, IIA/IIX, and I/IIA/IIX). In contrast to this dramatic alteration, repair of the spinal cord via PNG was partially effective in restoring the slow type I phenotype as evidenced by 1) a large pool (~20% of the total fiber population) of slow type I fibers and 2) substantial pools of I/IIA and I/IIA/IIIX hybrid fibers that contained large proportions of the slow type I MHC isoform.

BBB scores and correlation with slow type I MHC isoform expression. Six months following spinal cord surgery, hindlimb locomotion was evaluated using the BBB open-field test. The BBB scores for the sham control animals ranged from 20 to 21, and, as expected, the animals in the Tx group had very low BBB scores that ranged from 0 to 2 (Fig. 4). In contrast, five of the eight animals in the Tx+PNG group exhibited extensive movement (2 or 3 joints) in both hindlimbs, and the BBB scores of all animals in this group ranged from 3 to 8 (see Fig. 4). Compared with the Tx group, the Tx+PNG group had significant improvement of hindlimb locomotion (\( P \leq 0.01 \); Fig. 4A).

To determine whether there was a significant correlation between BBB score and the expression of the slow type I MHC isoform, we performed correlations between 1) BBB score and the percentage of pure slow type I fibers (i.e., monomorphic fibers; see Fig. 4B) and 2) BBB score and the percentage of fibers expressing any degree of the slow type I MHC isoform (i.e., mono- plus polymorphic fibers; see Fig. 4C). As shown in Fig. 4, Band C, there were strong correlations for both types of comparisons (i.e., \( r^2 > 0.9 \)).
Regrowth of 5-HT-positive fibers and correlation with slow type I MHC isoform expression. The immunoreactivity of 5-HT-positive fiber was used to investigate the regrowth of descending motor pathway in spinal cord injury. There were no 5-HT-positive fibers in or distal to the gap in the Tx animals. In contrast, 5-HT-positive fibers were found in the graft site and caudal to the graft site (Fig. 5, A–D) in the Tx/H11001 PNG animals. As shown in Fig. 5, there was a significant correlation between the number of 5-HT-positive fibers (caudal region) and the expression of the slow type I MHC isoform.

DISCUSSION

The findings of the current study are unique in several respects. First, the results of this study demonstrate that PNG repair of the transected spinal cord is partially effective in restoring muscle mass of the soleus muscle, an important postural and locomotor muscle. Second, this is accompanied by partial restoration of the slow phenotype of the soleus muscle. Finally, the expression of the slow type I MHC isoform in the soleus may represent an important biomarker of recovery given the significant correlation between it and the BBB score.

One of the hallmarks of spinal cord injury is a loss of muscle mass as manifested by a reduction in cross-sectional area. In rodents, the soleus muscle is very sensitive to reductions of activation/loading (4, 10), and this results in rapid atrophy. For instance, Talmadge et al. (23) reported that rat soleus muscle mass was reduced by \(-40\%\) following 15 days of spinal cord transection. Thereafter, there appears to be little change in rat


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soleus muscle mass. In the current study, we also observed that spinal cord transection produced a ~40% reduction in muscle mass (see Table 1), and this was associated with a large reduction in muscle fiber cross-sectional area (see Fig. 1). To date, a number of studies (2, 7, 11, 16, 20, 25) have used the PNG procedure; however, none of these studies assessed the effectiveness of this procedure in restoring muscle mass. In this context, we observed that the PNG procedure partially restored muscle mass by ~20% and resulted in muscle fiber cross-sectional areas that were ~40% larger than those seen in the TX soleus muscle. As described below, the partial recovery of muscle mass and fiber cross-sectional area were associated with a partial restoration of the slow MHC isoform phenotype. In future studies, it will be interesting to determine whether the recovery of muscle fiber cross-sectional area is specific to those fibers that recover their slow phenotype or whether the recovery of cross-sectional area is unrelated to slow type I MHC isoform expression.

As shown in Fig. 3, spinal transection produces a rather dramatic alteration in MHC isoform expression in the soleus muscle, and, to our knowledge, there is no other hindlimb muscle that appears to be as sensitive to altered activation/loading patterns. In the current study, we observed that ~90% of the fibers in the control soleus muscle expressed only the slow type I MHC isoform and that 6 mo following spinal cord transection, very few fibers (~1–2%) retained this slow phenotype. Hence, the original pool of slow type I fibers underwent MHC isoform transitions such that these fibers 1) only expressed the fast type IIA or fast type IIX MHC isoforms or 2) exhibited a polymorphic pattern of MHC isoform composition. Importantly, it should be stressed that ~50% of the fibers exhibited a polymorphic MHC isoform profile. In general, our findings are consistent with those published previously by Talmadge et al. (24). Although our analyses were performed at a single time point (i.e., 6 mo) following Tx, the time-course analyses performed by Talmadge et al. (23) demonstrate that MHC isoform transitions in the slow type I fibers occur rapidly (i.e., within 15–30 days) and that beyond these time points, large pools of hybrid fibers persist. The rapidity of these changes in MHC isoform expression suggests that the PNG repair of spinal cord transection restores phenotype rather than maintains it.

Given the sensitivity of the single-fiber MHC isoform composition of the soleus muscle to altered activation/loading states, we were interested in determining whether repair of spinal cord transection via PNG is effective in partially or completely restoring the MHC isoform profile of single fibers in the soleus muscle. In this context, our findings are unique and suggest that PNG repair can be partially effective in restoring the slow phenotype of the soleus muscle via 1) a significant recovery in the proportion of fibers that express the slow type I MHC isoform only (i.e., slow type I fibers) and 2) the large percentages (~70%) of hybrid fibers (i.e., I/IIA or I/IIA/IIX) that express high proportions of the slow type I MHC isoform. For instance, in the I/IIA/IIX pool of fibers, the slow type I MHC isoform accounts for ~50% of the total MHC. Collectively, when the expression of the slow type I MHC isoform in the various fiber types is summed across all of the fibers sampled, it represents ~50% of the total MHC pool. This is quite a contrast to the TX soleus muscles, in which the slow type I MHC at the whole muscle level was ~10% of the total MHC pool.

Given the findings discussed above, it is tempting to hypothesize that the MHC isoform composition of the soleus muscle may represent an important biomarker that reflects the extent of spinal cord repair and functional recovery in the PNG TX animals. As a first approximation for testing this concept, we examined the correlation between BBB scores and slow type I fibers (i.e., monomorphic) and 2) the percentage of fibers expressing the slow type I MHC isoform (i.e., mono- plus...
polymorphic proportions of fibers). In both instances, we observed very high coefficients of determination (i.e., $r^2 > 0.9$; see Fig. 4). Consistent with the idea that the slow MHC isoform phenotype may represent an important biomarker, linear regression analyses also demonstrated a good relationship between the proportion of slow type I fibers and 5-HT-labeled fibers below the level of transection. In addition to these observations, Golding et al. (12) observed a strong relationship between the relative slow type I MHC isoform and BBB scores ($r^2 = 0.87$) in soleus muscles of animals with spinal cord injury induced by clips. Although these correlative studies support the hypothesis that the MHC isoform profile of the soleus muscle can be a useful reporter of spinal cord recovery, rigorous studies are needed for providing a more stringent test of this concept and will provide more insight with respect to the significance of MHC isoform transitions observed in the current study. As noted above, PNG repair of TX spinal cords produced MHC isoforms transition that 1) resulted in the reappearance of pure slow type I fibers and 2) a transition from large proportions of fast IIA or IIX fibers to hybrid fibers that contained large proportions of the slow type I MHC isoform. It might be suggested that a normal activation/loading pattern was responsible for the reappearance of fibers expressing only the slow type I MHC isoform. Such supposition, however, requires further studies using approaches such as horseradish peroxidase labeling.

The restoration of the slow MHC phenotype also may reflect a potential modulatory influence of 5-HT-labeled fibers. In the current study, we observed that PNG repair was effective in promoting the growth of 5-HT-labeled fibers across the injury site. Importantly, it should be noted that previous investigators (22) have shown that serotonergic neurons can modulate motor function by increasing the excitability of motor neurons or by altering excitatory/inhibitory synaptic input to motor neurons. Consistent with this perspective, Cooper et al. (8) found that neuronal transplantation of serotonergic neurons from the raphe region was effective in partially restoring the percentage of slow type I fibers in the soleus.

**Conclusion.** In summary, the findings of this study are encouraging, because they demonstrate that PNG repair of transected spinal cords is partially effective in restoring muscle mass and MHC isoform phenotype. The slow MHC isoform profile of the soleus muscle is highly sensitive to altered activation/loading patterns, and it may represent an important biomarker that reflects the efficacy of techniques used to repair injured spinal cords. Further studies are required to rigorously test this concept and the importance of the reappearance of fibers that only express the slow type I MHC isoform. Finally, the reappearance of slow type I fibers in some ($\sim20\%$ of the total fibers) fibers suggests that reinnervation of the motor neuron controlling these fibers occurred, and, if true, then such a dichotomy in the same muscle may represent an important

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**Fig. 5.** Immunoreactivity of serotonin (5-HT) in longitudinal spinal cord section was used to determine the regrowth of descending fibers from the brain. *A:* illustration indicating the location of 5-HT-positive fibers. The photomicrographs demonstrate that 5-HT-positive fibers (arrows) were found in the graft site (*B*) and in the caudal host spinal cord 2 (*C*) and 10 mm (*D*) below the graft site. Note that there was a good correlation ($r^2 = 0.6831$) between the number of 5-HT fibers below the graft site and the percentage of slow type I fibers (*E*). A similar result ($r^2 = 0.5927$) was obtained when the number of 5-HT fibers was correlated with the proportion of fibers expressing some level of the slow type I MHC isoforms (*F*). Scale bar, 100 μm.
strategy for dissecting mechanisms underlying successful repair.

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